Comparative Studies on Isolation of Medium-chain-length Polyhydroxyalkanoates Produced by *Pseudomonas* spp. Strains

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Several methods were used for recovering medium-chain-length polyhydroxyalkanoates (mcl-PHAs) biosynthesized by a Pseudomonas spp. strain cells, in a fermentation medium based on sodium citrate and sodium octanoate. To improve bacterial cells separation, flocculation was tested, using a anionic polyelectrolyte. Post-biosynthesis processing of separated biomass included cell rupture and solubilization of all cell materials except PHAs and/or solvent extraction of PHAs. NaOCl or lysozyme + EDTA were used for biomass digestion, $CHCl_3$ and acetone for polyester extraction. The isolated polymers were characterized by GC-FID, FTIR, and DSC-TGA. The best results were obtained using acetone Soxhlet extraction from dried cells. Thus, mcl-PHAs were recovered as thin polymeric membranes, of above 99% purity, representing 44-54% of dry bacterial cells, and containing C6, C8 and C10 hydroxyacid monomers in a gravimetric percentage of: 9.50 -11.47% C6, 85.25 - 86.75% C8 and 1.85 - 3.01% C10.

Keywords: Medium chain length polyhydroxyalkanoates; Pseudomonas spp., extraction

Poly(β-hydroxyalkanoic acid)s (PHAs), also known as polyhydroxyalkanoates or microbial polyesters, are a class of natural thermoplastic polymers [1, 2]. Due to their properties, similar to those of conventional plastics and to their biodegradability, they have attracted much interest as alternatives to synthetic polymers, the more so as they can be produced from renewable resources and processed with the aid of equipments used for polyolefins or other synthetic polymers. The mechanical properties of PHAs depend on monomer structure and on molecular weight of polymers, varying between rubber like elasticity and brittleness of crystalline textolite [3]. PHAs (table 1) are specifically produced by a wide variety of bacteria, as an intracellular energy reserve, in the form of homo- or copolymers of [R]-â-hydroxyalkanoic acids, depending on the C source used for microorganism growth, when the cells are grown under restrictive conditions [4]. Depending on the number of carbon atoms contained by the monomers units, PHAs isolated up to now can be classified as follows (table 1): (i)short chain length (*scl*) PHAs - 3 to 5 carbon atoms/monomer, (ii) medium chain length (*mcl)* PHAs - 6 to 14 carbon atoms/monomer, and *scl-co-mcl* with repeat-unit monomers containing 3 to 14 carbon atoms [5].

As these biopolymers are biodegradable [5] and biocompatible, they are suitable for many applications, which can be divided in three main directions: packaging materials, replacing the oil-derived ones [5], biomedical materials useful in surgical sutures and tissue engineering [6, 7] and drug carriers in drug delivery systems [8].

The researches on obtaining PHAs with different compositions are continuously performed worldwide, at 3 levels: (i) improvement of biosynthesis capacity of producing microorganisms from a large range of substrates, mainly by genetic and metabolic engineering, (ii) bioprocess optimization and (iii) down-stream processing, isolation and purification improvement [9].

According to the published studies on this subject, the downstream processing of fermentation media containing PHAs is based on three types of procedures: (i) direct solvent extraction of the polymer from the mixture with the other cellular components [10]; (ii) chemical or biochemical disintegration of the cell wall and water extraction of all cell components except PHAs [11]; (iii) combinations of the above mentioned methods [12]. Solvent extraction implies PHAs preferential dissolution among the other cell components. The best solvents for

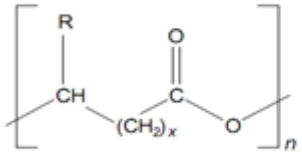
PHAs general formula	Type	*x*	R	Polymer name
	*scl*	1	H	Poly-(3-hydroxypropionate)
	scl	1	CH_3 –	Poly-(3-hydroxybutyrate)
	scl	1	$CH_3 – CH_2$ –	Poly-(3-hydroxyvalerate)
	scl	2	H	Poly-(4- hydroxybutyrate)
	scl	3	H	Poly-(5- hydroxyvalerate)
	mcl	1	$CH_3 – CH_2 – CH_2$	Poly-(3-hydroxyhexanoate)
	mcl	1	$CH_3 – (CH_2)_3$ –	Poly-(3-hydroxyoctanoate)
	mcl	1	$CH_3 – (CH_2)_7$ –	Poly-(3-hydroxydodecanoate)

Table 1
PHAs TYPES

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scl-PHAs extraction are the halogenated hydrocarbons: $CHCl_3$, CH_2Cl_2, CH_2Cl-CH_2Cl, CH_2Cl-CH_2Cl-CH_3. Other solvents mentioned as useful for PHAs recovery, depending on polymer composition, are the following: hexane, C3-alcohols and their acetates, such as isoamyl alcohol and isoamyl acetate, ketones, toluene, cyclic esters of carboxylic acids, methyl or ethyl lactate, acetic acid etc. [13].

Our study on *mcl*-PHAs isolation aimed to compare the performances of different methods in terms of recovery yield and polymer purity. The downstream processing of the fermentation medium included: cell rupture and solubilization of all cell materials except PHAs, solvent extraction of PHAs, and various combinations thereof. The obtained *mcl*-PHAs were analyzed by FTIR, differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA) to point out the influence of microbial synthesis on their structure and thermal behaviour.

Experimental part

Materials

The fermentation medium with bacterial cell mass containing 18 - 50 % *mcl*-PHAs was obtained by cultivating a strain of *Pseudomonas fluorescens* ICCF 392 (from ICCF culture collection of micro-organisms), in conditions previously reported [14]. Organic solvents, reagents and mineral salts were purchased from Merck. Methyl esters of 3-hydroxy acids (C6, C8, C9, C10, C11, C14, C16, C18), purity 98%, were purchased from Larodan Fine Chemicals, Sweden. Ponilit GT1 (an anionic water soluble polyelectrolyte, having a flocculation effect) was obtained from the Institute for Macromolecular Chemistry, Iasi, Romania. Polyhydroxybutyrate/polyhydroxyvalerate (PHBHV), a copolymer with 2% polyhydroxyvalerate produced by microbial synthesis, with a density of 1.25 g/cm^3 and maximum particle size of 300 μm, was purchased from Goodfellow Cambridge Ltd, UK and used as a reference.

Procedures and methods

Biomass separation

At the fermentation end, the cultivation medium pH value was adjust at 6 and Ponilit GT-1 was added as to reach different concentrations in the fermentation medium (0.01 - 0.05% v/v). The suspension resulted after Ponilit GT-1 adding was stirred for 5 min, let for sedimentation until the supernatant clarified, and the sedimentation time was registered for every flocculant concentration. Sediment particles of bacterial cells were separated by centrifugation at 4000 rpm, in a centrifuge (Hettich – Germany), washed with 2 volumes of physiological saline and the obtained

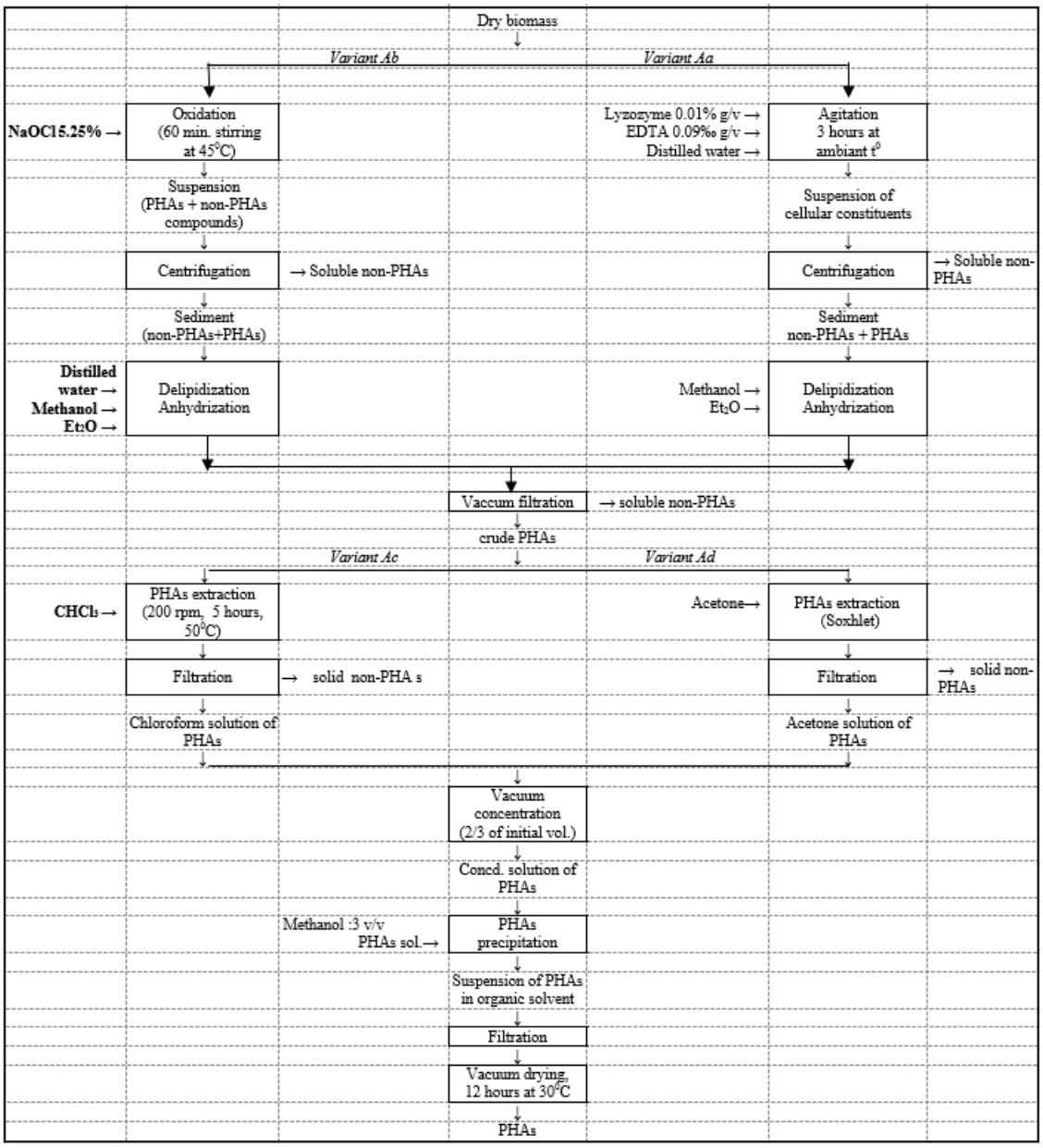

Fig. 1. Experimental variants for mcl-PHAs isolation from digested bacterial biomass

suspension was centrifuged again, in the same conditions. The supernatant was removed and the sediment biomass was dried at constant weight. The amount of dry biomass was also registered for every flocculant concentration.

mcl-PHAs recovery from bacterial cells

For PHAs extraction and isolation, the following combined methods were used (fig. 1): *mcl*-PHAs isolation after chemical or enzymatic biomass digestion, including: cell wall biochemical (Aa variant) or chemical (Ab) disintegration, delipidization and anhydrization of PHAs containing insoluble material by treatments with appropriate solvents, PHAs extraction in chloroform (Ac variant) or in acetone (Ad variant); for the AbAc processing variant (fig. 1), a ratio of 1:100:33 g/v/v dry biomass: NaOCl (12% active chlorine):$CHCl_3$ was used, or a simultaneous treatment with NaOCl (12% active chlorine) and $CHCl_3$ in different proportions.

Another experimental recovery procedure of PHAs consisted in acetone Soxhlet extraction of polymer without previous digestion of biomass. In this case a preliminary methanol treatment of dried bacterial biomass was performed, in order to dissolve certain non PHAs components, susceptible to be further extracted in acetone. Methanol was added to the dried biomass at a ratio of 20:1 (v/g); the resulted suspension was stirred 30 min at room temperature, and then centrifuged; the sediment was washed with distilled water and vacuum dried. Soxhlet extraction with acetone was conducted at 55-57°C, during 5 hours. The PHAs containing acetone solution was filtered (for removing bacterial cell components carried by acetone vapors) and concentrated at 2/3 from the initial volume. Methanol was added to the concentrated acetone and PHAs precipitated as glue was dissolved in chloroform. The polymer solution in chloroform can be further processed in two ways, depending on the PHAs initial content of biomass: slow evaporation at room temperature, in case of a polymer from a biomass with an initial high content of PHAs (40 - 60%) or a second precipitation with cold methanol (10 volumes to 1 volume of chloroform solution), with stirring, at + 4°C, over night, filtering, re-dissolving in chloroform and slow evaporation to dryness, in case the polymer is obtained from a biomass initially containing less than 20% PHAs.

PHAs assay: *mcl*-PHAs, obtained as a polymer pellicle, was measured by gas chromatography, after a mild acid methanolysis [15]. The polymer composition was expressed as monomer gravimetric percentage. For the monomer composition assessment, a capillary column has been used with a HP 5 (5% phenyl - methyl-polysiloxane) stationary phase. Methyl esters of C4 – C18 hydroxyacids have been used as standard substances.

Characterization of PHAs pellicles: *mcl*-PHAs pellicles were analyzed by Attenuated Total Reflectance Fourier Transform Infrared (ATR-FTIR) using a spectrometer TENSOR 37 from Bruker with the Golden Gate Single Reflection Diamond ATR accessory. Data were collected at room temperature from 4,000 to 1000 cm^{-1} with 16 scans at a resolution of 4 cm^{-1}. Reproducibility was confirmed for each sample by repeating the experiment 3 times. TGA analysis of mcl-PHAs pellicles was performed on TA-Q5000 V3.13 (TA Instruments Inc., USA) using nitrogen as the purge gas at a flow rate of 40 mL/min. The thermograms were acquired between 25 and 700 °C at a heating rate of 10 °C/min. For each measurement duplicate samples weighing between 8 and 10 mg were used.

DSC experiments on mcl-PHAs pellicles were performed on a SDT Q600 V20.9 from TA Instruments under helium flow (100 mL/min). The samples weighing around 8 mg were packed in aluminium pans, as above mentioned. The melting temperature (T_m) was taken as the peak temperature of the melting endotherm.

Ponilit GT-1 concentration (%v/v)	Sedimentation time (min)	Recovered dry biomass (g)
-	30	0.32
0.1	10	0.33
0.2	5	0.33
0.5	3	0.33

Table 2
INFLUENCE OF PONILIT GT1 CONCENTRATION ON PHAs CONTAINING CELL SEDIMENTATION

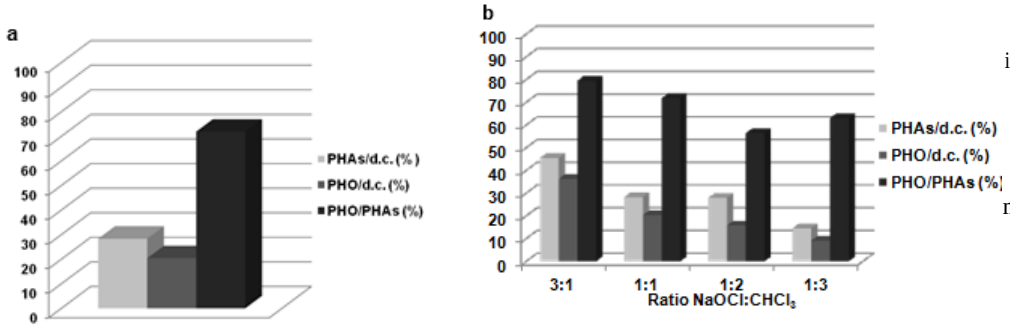

Fig. 2. (a) mcl-PHAs (PHO) isolation by enzymatic digestion of biomass followed by $CHCl_3$ extraction (AaAc variant); (b) Influence of NaOCl:$CHCl_3$ ratio of the extraction mixture on mcl-PHAs (PHO) isolation (AbAc variant with simultaneous digestion and extraction treatment)

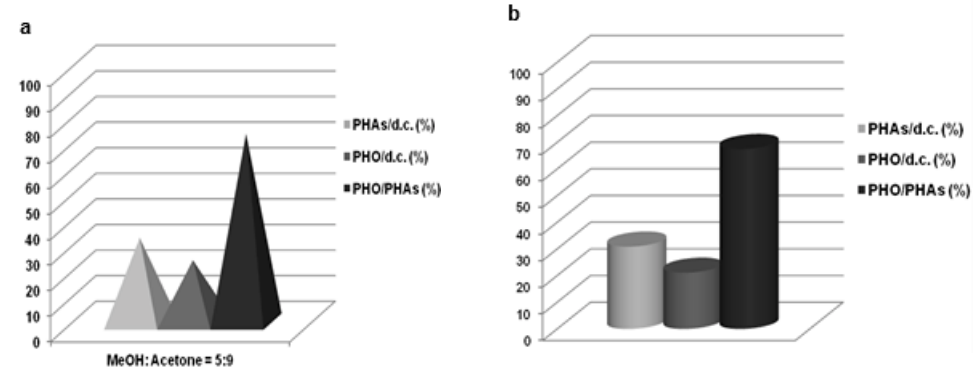

Fig. 3. *mcl*-PHAs (PHO) isolation by (a) enzymatic digestion followed by acetone extraction (AaAd variant); (b) NaOCl treatment followed by acetone extraction (AbAd variant)

Table 3
EXAMPLES OF COMPOSITIONS OF PHAS OBTAINED AFTER CHEMICAL OR ENZYMATIC DIGESTION OF MICROBIAL BIOMASS FOLLOWED BY SOLVENT EXTRACTION

PHAs (g/L)	PHAs % (d.c.)	Hydroxyacids					
		C6(%)	C8(%)	C10(%)	C11(%)	C14(%)	Molar ratio C6:C8:C10:C11:C14
0.6405	31.42	15.83	71.90	0.72	1.25	0.47	11:45:0.4:0.6:0.2
0.2212	18.08	13.63	63.08	0.69	2.67	0.67	9:40:0.4:1.3:0.3
0.5834	39.01	11.70	56.46	0.90	2.20	0.57	8:35:0.5:10:0.2
0.8414	28.17	16.68	73.46	0.78	0.49	-	10.6:49:0.5:0.2

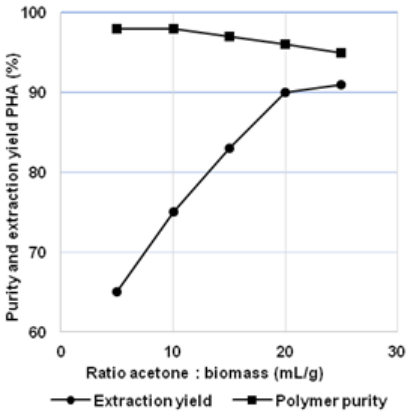

Fig 4. Influence of acetone: biomass ratio on *mcl*-PHAs (PHO) extraction yield and purity

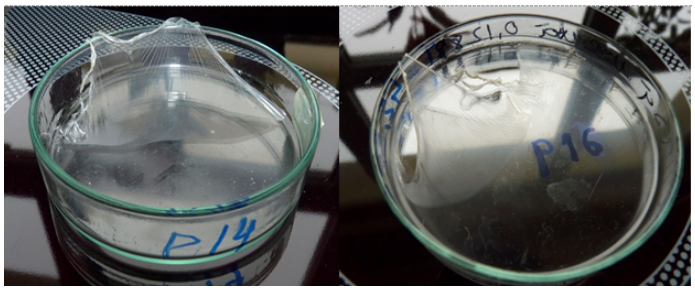

Fig.5. *mcl*-PHAs pellicles obtained from *Pseudomonas* spp.

Table 4
ANALYTICAL CHARACTERISTICS OF POLYMERS RECOVERED BY SOXHLET ACETONE EXTRACTION

mcl-PHAs sample	PHAs (g/L)	PHAs % (d.c.)	Hydroxyacids					
			C6(%)	C8(%)	C10(%)	C11(%)	C14(%)	Molar ratio C6:C8:C10:C11:C14
1	1.055	38.85	10.98	85.25	2.70	-	-	8:51:1.3
2	0.977	43.76	9.50	86.42	3.01	0.30	-	7:52:1.5
3	1.490	53.69	11.47	85.75	1.85	-	-	9:54:1.0
4	1.327	44.03	10.05	86.75	2.08	0.3	-	7:50:0.4:0.1

Results and discussions

PHAs containing biomass separation using Ponilit GT1

In the experiments carried on to establish the optimum concentration of the flocculating agent, samples of 100 mL fermentation medium were used and treated with the same volume of Ponilit GT1 solutions of different concentration. The results obtained in these experiments, presented in table 2, showed that in all cases (meaning Ponilit GT1 concentration between 0.1 and 0.5% mL/100 mL fermentation medium), the flocculating agent presence considerably reduces the cell sedimentation time (3 to 10 time shorter), depending on flocculant concentration) and in all cases allows the complete recovery of biomass from the fermentation liquid.

PHAs extraction after chemical / biochemical biomass digestion

The following experimental variants (presented in fig. 1) were carried on: *(AaAc)* - enzymatic digestion (lysozyme + EDTA), followed by chloroform extraction: $CHCl_3$-/ sediment (non PHAs + PHAs) = 33:1; *(AbAc)*: chemical digestion (NaOCl), followed by chloroform extraction; *(AaAd)*: enzymatic digestion, followed by acetone extraction; *(AbAd):* chemical digestion, followed by acetone extraction.

The polymers obtained through each of the above variants were analyzed by GC-FID and FTIR, and the results displayed in the following figures show the *mcl*-PHAs and poly-(3-hydroxyoctanoate) (PHO) quantities from 100 g processed dry cells (d.c.), as well as the ratio PHO:*mcl*-PHAs.

Comparatively analyzing these experiments, in terms of percentage of PHAs recovered from dry cells and their content in PHO, it came out that the best results were obtained in AbAc variant, meaning consecutive treatments of digestion and extraction (with NaOCl and, respectively, $CHCl_3$, at a ratio of 3:1 v/v, 2 h, with stirring, at 40 – 45°C. Separating the phases and evaporating to dryness the organic phase, polymer films were obtained, at a ratio of 30.85% - 45.58 % from dry biomass, with a C4 – C18 content of 72 - 91.5%, in which C8 is found in preponderant ratios, between 56.5 and 73.5%.

Soxhlet acetone extraction of PHAs without preliminary biomass digestion

For this type of extraction, dry biomass was used after a methanol treatment for removing impurities susceptible to be extracted in acetone. This set of experiments led to an optimal value of 1:10 for the dry cells: acetone ratio (g/v), which was pointed out as allowing the best correlation between the extraction yield (90%) and the polymer purity (97%), as it is presented in figure 4.

As in the case of the PHAs isolated by two-steps biomass processing (digestion followed by solvent extraction) the composition and purity of polymers isolated only by Soxhlet extraction with acetone were determined by GC-FID and expressed in gravimetric percentages (table 4).

Unexpectedly, the analytical results show that applying Soxhlet acetone extraction, polymers with a higher content

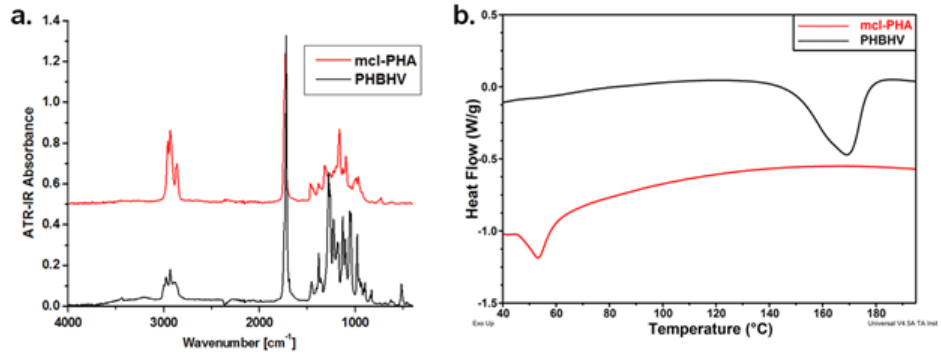

Fig. 6. (a) FTIR spectra of *mcl*-PHAs and PHBHV; (b) DSC thermograms of *mcl*-PHAs and PHBHV

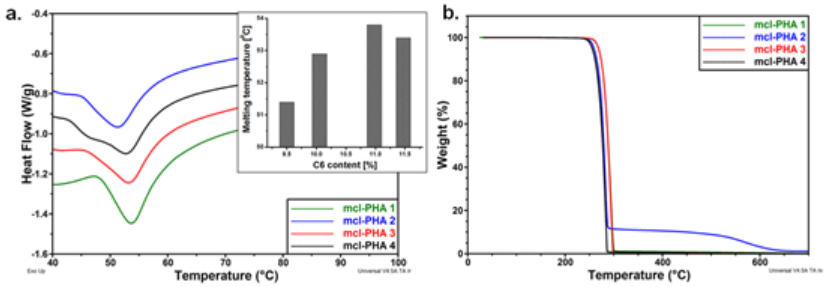

Fig. 7. (a) DSC thermograms of *mcl*-PHAs (inset: Melting temperature vs. C6 content in PHAs); (b) TGA curves of *mcl*-PHAs

mcl-PHAs sample	Melting temperature (°C)	Melting enthalpy (J/g)	On-set degradation temperature (°C)
1	53.8	13.6	279
2	51.4	11.2	270
3	53.4	12.2	280
4	52.9	15.1	270

Table 5
THERMAL CHARACTERISTICS FOR *mcl*-PHAs SAMPLES

in C8 monomers, ranging between 85.25 to 86.75% (comparative with the other isolation method tested) , C6 between 9.5 and 11.47 % and C10 between 1.85 and 3.01%, were obtained. An image of PHAs pellicles containing 85-87% C8 monomers, obtained from *Pseudomonas* spp. is shown in figure 5.

Characterization of PHAs pellicles

\FTIR spectrum of *a mcl*-PHAs pellicle is given in figure 8a and, for comparison, the FTIR spectrum of PHBHV (commercial grade). *mcl*-PHAs are not yet produced at industrial level and the comparison with a marketed product, with known characteristics, is important for defining their structure. PHBHV shows the main absorbance peaks at 2975 cm^{-1} and 2932 cm^{-1}, characteristic to asymmetric and symmetric stretching vibration of CH_3, at 1720 cm^{-1}, the strong stretching vibration of C=O, and the bands in the range from 1280 cm^{-1} to 1060 cm^{-1}, which correspond to C–O–C stretching vibrations. The most important peaks characteristic to CH_3 and C=O stretching vibrations also appear in the case of *mcl*-PHAs pellicle, but slightly modified as intensity and wavelength. The bands for CH_3 stretching vibrations are slightly shifted in *mcl*-PHAs, compared to PHBHV, at 2957 and 2931 cm^{-1}, and that of C=O at higher wavelength, 1725 cm^{-1}. It should be remarked the higher intensity of C-H stretching vibrations in *mcl*-PHAs compared to PHBHV, as result of the increased CH_2 chain length. Moreover, the shift of C=O vibrations at higher wavelength in *mcl*-PHAs compared to PHBHV suggest a different ratio of amorphous/crystalline phases in the two samples [16].

The higher content of amorphous phase in *mcl*-PHAs compared to PHBHV was verified by DSC. DSC thermograms of the two polymers from figure 8b show different thermal behavior, much lower melting temperature for *mcl*-PHAs compared to PHBHV (53°C instead of 169°C). *mcl*-PHAs behave as thermoplastic elastomers because of the higher CH_2 chain length in their structure, which hinders the hydrogen bonding, leading to higher mobility of the chains and lower melting temperature. Moreover, *mcl*-PHAs show lower melting enthalpy (14 J/g instead of 56 J/g) and, therefore, higher amorphous phase/crystalline phase ratio, which confirms the FTIR results.

The thermal behavior of four *mcl*-PHAs samples obtained by Soxhlet acetone extraction was characterized by DSC (fig. 7a) and TGA (fig. 7b).

DSC diagrams show some influence of monomer composition on the thermal behavior. Characteristic data were cumulated in table 5. All the tested samples show melting temperature in the range from 50 to 54°C and melting enthalpies from 11 J/g to 15 J/g, so small crystallinity degree. It seems that C6 content has some influence on the melting behavior, lower C6 fractions in PHAs being related to smaller melting temperature values (fig. 7a-inset).

TGA curves of tested PHAs indicate one-step degradation process for almost all the samples, with the exception of *mcl*-PHAs sample no. 2, which showed a two-step degradation process. The on-set degradation temperature is higher than 270°C for all the samples. Once more the samples with higher C6 content (samples no. 1 and 3) show higher on-set degradation temperature (with 9 and 10°C, respectively – table 5), so better thermal behavior than that with lower C6 content (samples 2 and 4).

Conclusions

Considering the accumulation of PHAs in the cytoplasm, in the form of granules insoluble in physiological medium, the first stage of post-fermentation processing has to be the separation of biomass from the fermentation liquid, which was improved with the aid of a flocculating agent. Further, several steps of downstream processing were carried on and compared: disintegration of cell walls by chemical/biochemical digestion of biomass, removal the

non-PHAs solid components using suitable solvents, extraction of PHAs in chloroform and/or acetone, combinations of these methods.

The best results obtained for the isolation method based on two-steps biomass processing (digestion followed by solvent extraction), were registered in case of the consecutive treatment with NaOCl, and $CHCl_3$. Organic phase evaporation to dryness led to polymer films (30.85% - 45.58 % from dry biomass), containing 72 - 91.5% C4 – C18, in which C8 is found in ratios between 56.5 and 73.5%.

Soxhlet extraction with acetone of PHAs form undigested dry biomass, but pretreated with methanol, unexpectedly lead to even better results, represented by polymers containing (in gravimetric percentages) from 9.5 to 11.47 C6, from 85.25 to 86.75 C8 and from 1.85 to 3.01 C10, with polymer recovery yields of 90%.

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